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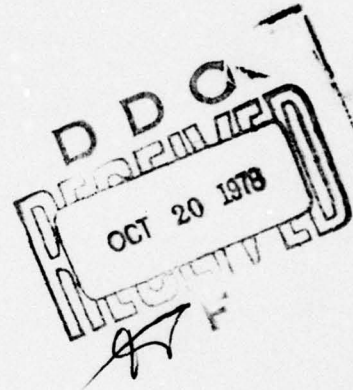
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THE EFFECT OF LASER ENERGY ON CELLS IN TISSUE CULTURE

Final Report

Donald E. Rounds, Ph.D.

May, 1978



Supported by

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The first area reported that energy-dependent biological systems, such as mitosis and contractile activity of heart, skeletal muscle and smooth muscle tissues, showed a temporary (and sometimes permanent) reduction in activity when irradiated with a ruby laser. When these rate changes were correlated with the observations that cellular ATP was not utilized and that ATPase activity, in the form of luciferin-luciferase, was inhibited, we concluded that biological activity was slowed or stopped because energy was not released from stores of ATP.

Evidence for another indirect form of biological damage stemmed from the observation that ruby laser irradiation of cells in tissue culture depleted the population of intact lysosomes and released a mixture of amino acids, nucleosides and nucleotides. This mixture, as well as one of its constituents, ADP, when administered at appropriate concentrations, could stimulate cell growth by 12-15%, agglutinate erythrocytes, promote blood clotting rate and induce white blood cell chemotaxis. These data suggest that laser-induced lysosomal lability resulted in a series of complex events, some of which could produce inflammatory or toxic reactions in tissues.

Studies utilizing a variety of laser wavelengths illustrated the requirement for absorbing laser energy in natural or artificial chromophores before biological effects could be produced. Pigmented cells were damaged by ruby laser treatment in proportion to the number of melanin granules per cell. Erythrocytes were bleached by a frequency-doubled neodymium (Nd) laser operating at 5300A, but the same power density from a ruby laser showed no effect. Electron transport was blocked between cytochromes b and c when the latter chromophore was treated in brain tissue with laser power at 5300A. Similarly, wavelengths of 6096 and 6013A from a Raman-shifted ruby laser beam was seen to selectively reduce the activity of cytochrome a as an electron acceptor. Enzymatic activity of lactic dehydrogenase (LDH) was not inhibited by a laser wavelength of 3472A unless it was treated in the presence of an appropriate chromophore, reduced nicotinamide adenine dinucleotide (NADH). However, LDH was directly affected by a laser wavelength of 2650A from a frequency-quadrupled Nd laser. DNA template activity was also inhibited by laser irradiation at 2650A, due to its matching absorption characteristic. Intact cells, when treated with this individual UV laser-treated cells showed each of the recognized forms of chromosome and chromatid aberrations. In contrast, the primary wavelength from the Nd laser (10,600A) had no effect on the growth rate of human diploid fibroblasts in cell culture, nor on their DNA, RNA or protein synthesis rates.

The fourth area of study demonstrated that the high photon density in a ruby laser beam could produce biochemical and biological changes through the process of two photon absorption. When two photons at 6943A were absorbed by target molecules simultaneously, its effect was equivalent to the absorbance of a single photon at 3472A. Thus high photon densities from a ruby laser excited fluor-

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Abstract - Continued

escence in a solution of NADH and inhibited its co-enzyme activity. In contrast to the response from pigmented rabbits, the iridial sphincter muscle from albino rabbits showed laser-induced contraction amplitudes which would be expected from the two photon absorption process.

It was concluded that laser energy, when absorbed directly or by the two photon absorption process, could produce a number of biological and biochemical events. Some of the effects were considered to be due to a lack of energy release from ATP or to the catabolic effects of released lysosomal acid hydrolases. Not all laser-induced damage is caused by thermal denaturation or charring.

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SUMMARY

The final report, covering a period of thirteen years of research, summarizes four areas of activity which helps define the mechanisms of biological damage produced by various energy densities, pulse widths and wavelengths from laser radiation.

★ The first area reported that energy-dependent biological systems, such as mitosis and contractile activity of heart, skeletal muscle and smooth muscle tissues, showed a temporary (and sometimes permanent) reduction in activity when irradiated with a ruby laser. When these rate changes were correlated with the observations that cellular ATP was not utilized and that ATPase activity, in the form of luciferin-luciferase, was inhibited, we concluded that biological activity was slowed or stopped because energy was not released from stores of ATP.

★ Evidence for another indirect form of biological damage stemmed from the observation that ruby laser irradiation of cells in tissue culture depleted the population of intact lysosomes and released a mixture of amino acids, nucleosides and nucleotides. This mixture, as well as one of its constituents, ADP, when administered at appropriate concentrations, could stimulate cell growth by 12-15%, agglutinate erythrocytes, promote blood clotting rate and induce white blood cell chemotaxis. These data suggest that laser-induced lysosomal lability resulted in a series of complex events, some of which could produce inflammatory or toxic reactions in tissues.

★ Studies utilizing a variety of laser wavelengths illustrated the requirement for absorbing laser energy in natural or artificial chromophores before biological effects could be produced. Pigmented cells were damaged by ruby laser treatment in proportion to the number of melanin granules per cell. Erythrocytes were bleached by a frequency-doubled neodymium (Nd) laser operating at 5300A, but the same power density from a ruby laser showed no effect. Electron transport was blocked between cytochromes b and c when the latter chromophore was treated in brain tissue with a laser power at 5300A. Similarly, wavelengths of 6096 and 6013A from a Raman-shifted ruby laser beam was seen to selectively reduce the activity of cytochrome a as an electron acceptor. Enzymatic activity of 3472A unless it was treated in the presence of an appropriate chromophore, reduced nicotinamide adenine dinucleotide (NADH). However, LDH was directly affected by a laser wavelength of 2650A from a frequency-quadrupled Nd laser. DNA template activity was also inhibited by laser irradiation at 2650A, due to its matching absorption characteristic. Intact cells, when treated with this wavelength showed reduced DNA and RNA synthesis rates. In addition, individual UV laser-treated cells showed each of the recognized forms of chromosome and chromatid aberrations. In contrast, the primary wavelength from the Nd laser (10,600A) had no effect on the growth rate of human diploid fibroblasts in cell culture, nor on their DNA, RNA or protein synthesis rates.

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Summary - Continued

★ The fourth area of study demonstrated that the high photon density in a ruby laser beam could produce biochemical and biological changes through the process of two photon absorption. When two photons at 6943A were absorbed by target molecules simultaneously, its effect was equivalent to the absorbence of a single photon at 3472A. Thus, high photon densities from a ruby laser excited fluorescence in a solution of NADH and inhibited its co-enzyme activity. In contrast to the response from pigmented rabbits, the iridial sphincter muscle from albino rabbits showed laser-induced contraction amplitudes which would be expected from the two photon absorption process.

★ It was concluded that laser energy, when absorbed directly or by the two photon absorption process, could produce a number of biological and biochemical events. Some of the effects were considered to be due to a lack of energy release from ATP or to the catabolic effects of released lysosomal acid hydrolases. Not all laser-induced damage is caused by thermal denaturation or charring.

Foreward

In conducting the whole animal portion of the research described in this report, the investigators adhered to the "Guide for Laboratory Animal Facilities and Care", as promulgated by the Committee on the Guide for Laboratory Animal, Resources, National Academy of Sciences - National Research Council.

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I. Introduction

When the contract period was initiated, few laser systems had been made to operate and few observations of biological reactions to laser energy had been reported. Therefore, a broad survey was made to determine if measurable responses could be observed in a variety of tissue culture systems following exposure to pulsed and Q-switched ruby lasers. Preliminary data obtained from this survey provided the basis for four major areas of research activity throughout the remainder of the contract period. These research areas were: (1) the effect of ruby laser energy on energy-dependent systems, (2) the effect of laser-induced heat on lysosomal lability, (3) wavelength-specific laser damage, and (4) a demonstration that some biological effects could be attributable to the two-photon absorption phenomenon. This report will summarize the experimental evidence used to define these fundamental mechanisms of laser-induced damage in biological tissues.

II. Ruby Laser Energy Effects of Energy-Dependent Systems

A. The Effect on Mitosis

An established line of human adenocarcinoma cells was irradiated with a total of 20 j/cm^2 . The cells were fixed for evaluation of the mitotic index after one hour. Where unirradiated control populations showed a total of 19 prophase figures in 6000 cells scored, the irradiated population showed 46 prophase figures out of 6000 cells. Therefore, a delay in the duration of prophase was noted in these random populations.

Salamander lung cells were irradiated with a focused beam which provided an energy density of 1000 j/cm^2 . The irradiation of either prophase or metaphase figures stopped the process of mitosis without apparent injury to the chromosomes. The chromosomes eventually became diffuse and formed new nuclear membranes. This process was observed to be analogous to treatment of the same cell type with colchicine, which prevents the formation and function of spindle fibers.

B. The effect on the beat frequency of chick heart muscle.

An excised heart, removed from a 3-day chick embryo, showed a rhythmic contraction rate of 166 beats per min.; following the exposure of this tissue to 2.5 j/cm^2 laser energy, the rate of contraction dropped to 116. An additional exposure produced a drop to 96 beats per min. After a 30 min. recovery period, the rate had increased to 148 beats per min. The heart muscle showed outgrowth of isolated cells during the two days following irradiation,

suggesting that no permanent damage had occurred. Tracings were made of the contraction patterns of this heart. The contractions occurring during the control period showed a simple wave form with a variable amplitude but a uniform frequency. This was decreased after the first exposure to laser energy. Following the second irradiation, the pattern showed secondary contractions superimposed on the primary movements, as well as reduced frequency. The recovery phase showed a partial return to the initial rate, and contraction patterns resembled those of the control condition.

Other hearts from 4-day chicks showed a response analogous to that described for the 3-day chick heart. Hearts from 5- and 6-day chicks showed no change of the atrial beat following laser irradiation, but the ventricular beat exhibited a reversible slowing response.

In order to demonstrate that muscle cells could be affected directly by laser energy, isolated myoblasts were obtained as test objects by trypsinizing chick hearts. These contractile elements showed variable rates of activity. Only those cells with rhythmic beat and showing rates greater than 70 contractions per min. were selected for study. The heart cells were monitored over a 15 min. period, for consistency in contractile activity, then exposed to 2.3 j/cm^2 laser energy. It was noted that the postirradiation activity was 70 to 80 percent of control values immediately after exposure. No recovery to pre-irradiation beating rates was found, but there was a progressive decrease in activity throughout the 3-hour observation period.

C. The effect on skeletal muscle contraction.

The leg muscle of 13-day chick embryos demonstrated rhythmic, spontaneous contractions after 1 week in tissue culture. On the 11th day of culture, the rate of these contracting elements was recorded on strip chart paper. An initial attempt to modify activity with a focused laser beam, at a dose of 600 j/cm^2 , resulted in complete tissue destruction. Subsequent exposures of contracting skeletal muscle strips, with only 6 j/cm^2 , showed a marked reduction in activity with the eventual loss of all movement. In a typical experiment, the relatively regular preirradiated contraction rate of 26 beats per min. was changed to a very irregular rhythm during the first minute after laser irradiation. This rhythm became more regular shortly thereafter but showed average values of 14, 6, and 0.5 beats per min. for the 5-, 30-, and 60-min. postirradiation periods, respectively. All movement ceased 70 min. after exposure to laser energy. It was also observed that adjacent myoblasts showed progressive cytoplasmic vacuolation during this period.

D. The effect on peristaltic movement of smooth muscle tissue

One cm. segments of large intestine from 16- to 18-day chick embryos were maintained for short periods as hanging drop preparations. A magnified image of this tissue on the view plate of a photomicrographic apparatus permitted observation and the recording of rhythmic peristalsis. Activity varied within these preparations from 1 to 3 contractions per min. but each intestinal segment showed a regular peristaltic rate during a 40-min. control period. Laser irradiation of the end of the segment, which showed the origin of the wave, resulted in a gradual disruption of the contraction rate. Periods between contractions became progressively lengthened, and no recovery was observed. Low energy densities (25 j/cm^2) decreased the contraction rate to 33 percent of control values, while a higher laser dose (1000 j/cm^2) produced complete cessation of organized movement within 20 min. after irradiation.

E. The effect on ATP metabolism

An established cell line (CMP) of human adenocarcinoma cells were subjected to 20 exposures of 1 joule each. ATP was extracted from these cells and from unirradiated control cells, using boiling distilled water. Analysis of the cellular ATP content, using the luciferin-luciferase system, showed that ruby laser-treated cells contained significantly more ATP than control cells.

When microdrops of luciferin-luciferase were exposed to 1000 j/cm^2 from a focused ruby laser beam, its ability to convert a measured amount of ATP to ADP was temporarily inhibited. This reproducible suppression in ATPase activity in solution suggested that the observed inhibition of mitotic activity and muscular contraction rates in living cells and tissues was the result of laser-induced ATPase inhibition.

III. The effect of laser-induced heat on lysosomal lability.

During the course of our studies, we had observed that ruby laser-treated endothelial cells growing in a tissue culture flask produced a substance in the culture medium which caused an increase in UV absorption in the culture medium. This observation led to a study of the chemical nature of the substance, as well as its biological activity.

It was established that both rabbit endothelial cells and an established line of human (CMP) cells could produce the chemical substance in question. This substance could be seen most readily in cells maintained for a few hours in balanced salt solution (BSS), in view of the fact that its ultraviolet absorption spectrum coincided with that of

many of the organic nutrients within the culture medium. The substance showed an absorption maximum at 2650A, it was dialyzable; and it was both ninhydrin and Lowry-positive. The Lowry-positive color reaction was not enhanced with cupric ions, suggesting that it was not a protein but was a smaller molecular weight amino acid or peptide. Separation of the extract on a sephadex column suggested that there were at least two components rather than one. One of these showed an absorption maximum at 2650A, and the other one had an absorption characteristic at 2750A. Thin-layer chromatography indicated that the substance contained three components when a chloroform-methanol-ammonium hydroxide buffer was used. The first component had an R_f value of 0.65. It showed a UV absorbant spot on the chromatography plate and was slightly ninhydrin-positive. The second component had an R_f value of 0.42. It was fluorescent when excited with a UV source. The third component had an R_f value of 0.18 and was strongly absorbent in UV light. It seemed possible that one of the components was serotonin and at least one other was an adenine nucleotide.

This material seemed to be produced by cells in culture as a result of a wide variety of non-specific types of trauma. These would include the exposure 50 j/cm² of ruby laser energy or heat shock at 42°C for two hours. The hypothesis that the factor was the result of acid hydrolases released from labilized lysosomes was supported by the observation that both 50 j/cm² of ruby laser energy and heat shock at 42°C for two hours caused depletion of acid phosphatase-positive granules in the cytoplasm of CMP cells. This observation suggested that cathepsins and nucleases, released from lysosomes, could cause a partial digestion of proteins and nucleic acids to produce a mixture of amino acids, purines and pyrimidines.

The constituents harvested from balanced salt solution had a number of biological activities. It was first observed that a suspension of human red blood cells placed in a low concentration of phytohemagglutinin were made to agglutinate over a period of about 30 minutes. If the constituents from traumatized cell populations were added to the suspension, the agglutination rate was accelerated to approximately 8-10 minutes. It was further observed that erythrocytes responded to an optimum concentration of this agglutinin and that either below or above this concentration the agglutination rate was diminished. The second example of biological or biochemical activity was the facilitation of human blood clotting rates. Blood mixed with an equal volume of saline clotted in an average of 14 minutes. Blood mixed with saline which contained the biological extract clotted at variable rates proportional to the concentration of the chemical constituents. With

high concentrations of the extract, clotting occurred in less than 2 minutes. Thirdly, the migration of human leukocytes harvested from the buffy coat layer of centrifuged blood cells was shown to demonstrate positive chemotaxis to the extract when introduced into a clot containing these cells. The migration of the leukocytes showed a preferential distribution toward the source of the extract, with the result of a higher cell density near that site. Finally, a strain of human skin fibroblasts were observed to show a 12%-15% stimulation in growth rate when treated with culture medium harvested from laser-irradiated cells.

An enzymatic test for AMP, ADP and pyruvic acid, was applied to aliquots of the BSS. Variable amounts of ADP (approximately $5 \times 10^{-5} \text{M}$) and pyruvate (5×10^{-5} to $5 \times 10^{-4} \text{M}$) were detected in different samples. In order to simplify the scope of this study, only one of these compounds (ADP) was tested for comparative biological activity. It was established that the addition of ADP in concentrations of 10^{-7}M and 10^{-6}M stimulated the growth rate of CMP cells 13% and 9%, respectively, above control values. The cell number in medium containing 10^{-5}M was equivalent to that of controls, while concentrations of 10^{-4}M , 10^{-3}M and 10^{-2}M ADP reduced the cell number to 67.5%, 45.5%, and 23.0% of control populations, respectively. This response mimicked that of 10-fold dilution increments of agents in medium from traumatized cells.

It is known that ADP can induce the aggregation and agglutination of platelets. The addition of BSS from traumatized cells to platelet-rich human plasma showed agglutinated platelet masses of a size and density which was equivalent to that produced by 10^{-3}M ADP. Besides platelet agglutination, it was observed that both ADP and the laser-induced factor promoted fibrin formation, in contrast to a relative lack of activity in control BSS. When ADP was added to diluted whole blood, it was determined that a critical range from $5 \times 10^{-4} \text{M}$ to $5 \times 10^{-3} \text{M}$ ADP produced a linear decrease in the clotting time to a minimum of 50% of control times. ADP concentrations less than $5 \times 10^{-4} \text{M}$ had no effect on blood clotting, while 10^{-2}M or greater prevented blood clot formation. This response compared favorably with the promotion of clotting by factors harvested from the medium following ruby laser treatment.

It was concluded, therefore, that low energy densities from a ruby laser could produce biological effects through an indirect pathway, namely: release of acid hydrolases from laser-induced lysosomal labilization, partial digestion of cellular macromolecules, and diffusion of biologically active catabolic products through the tissue surrounding the target area. One biologically active component of this mixture was found to be ADP. This concept was applied

to a re-evaluation of the threshold damage for CO₂ laser irradiated rabbit corneas, since the CO₂ laser is well known to produce heat which can labilize lysosomes.

A number of investigators have explored the effect of CO₂ laser energy on corneal epithelium of laboratory animals such as rabbits and monkeys. The criteria for damage was the production of a flat white area of edema, as indicated by corneal opacity, which disappeared within 24 hours.

However, it seemed possible that clarification of the corneal epithelium was not necessarily an indication of complete recovery of that tissue. Therefore, the goal of this study was to investigate the possibility of enzymatic damage, within the corneal epithelium, which persisted following exposure to threshold energy-density levels from the CO₂ laser.

The CO₂ laser used for all studies was the GTE Sylvania CO₂ laser, model No. 941 with a maximum of approximately 3 watts power output. The exposure time was controlled by a Uniblitz shutter and the power output was monitored with a Model 210 Power Meter from Coherent Radiation.

Young adult rabbits were anesthetized from intravenous injections of nembutal and placed in the path of the CO₂ laser beam. Immediately before exposure, the power meter was placed in front of the subject's eye in order to monitor the power output of the laser. The power meter was then removed from the path of the beam and the center of the cornea was exposed, using a predetermined power density and exposure time. Power densities in this study ranged from 5-20 W/cm². Exposure times ranged from 10-1000 msec.

At various time intervals after radiation, the rabbits were sacrificed and the corneas removed for analysis. The lactic dehydrogenase staining reaction was induced in whole corneas by incubating the cornea in a mixture of 45 mg lactate, 450 mg PVP, 11 mg NAD and 15 mg nitro-blue tetrazolium (NBT) in a final volume of 100 ml of 0.1 M Phosphate buffer (pH 7.2).

We confirmed that irradiation of previously reported threshold values of the rabbit's cornea could produce corneal opacity within 30 minutes; a phenomenon which would disappear within twenty-four hours. However, the 24 hour post-irradiation corneas which were examined for lactic dehydrogenase (LDH) activity showed an intense NBT staining reaction in all regions except for the target area. In this zone, the staining intensity (and therefore LDH activity) was greatly diminished in the 24 hour post-irradiation corneas.

By irradiating a series of corneas with varying energy densities and exposure times, we were able to establish

that the threshold for reducing LDH activity was at a power density which was 55% of that causing corneal opacity. When corneas were irradiated with a corneal opacity threshold dose, and examined at 0, 24, 48 and 72 hours after radiation, it was apparent that the area occupied by the reduced staining intensity increased in diameter up to twice the initial target diameter. This spreading phenomenon suggested a secondary mechanism of enzymatic damage which progressed in its effect beyond the initial laser irradiation event.

In order to determine if the response of the lactic dehydrogenase in the cornea was due solely to a temperature rise induced by the CO₂ laser or to some indirect effect, solutions of lactic dehydrogenase were irradiated as microdrops and their enzymatic reaction rates were measured immediately after irradiation. In order to duplicate the physical conditions of the LDH within the corneal epithelium, we maintained the initial drop at 32°C, the temperature reported to be present at the surface of the rabbit cornea. The laser irradiation was imposed at varying power densities and exposure times. In so doing, we established the threshold power density required to reduce the rate of enzymatic reaction below the non-irradiated or control rate for the same enzyme solution.

It was established that the threshold power density required to inactivate the LDH solution was 2.5 times higher than the threshold for LDH inactivation in the corneal epithelium. In addition, it was observed that this threshold was 1.3 times higher than the corneal opacity threshold. In view of the fact that a high threshold value was required for inactivation of LDH solutions and that the staining reaction for tissue LDH demonstrated a spread of the lesion diameter throughout a 72-hour post-irradiation period, it was concluded that another variable was influencing LDH inactivation within the whole tissue.

After applying the energy density threshold for producing corneal opacity, we sacrificed animals at 0, 24 and 48 hours in order to determine if changes in acid phosphatase activity could occur within the target area with respect to the non-irradiated portions of the cornea. Changes in the staining reaction for acid phosphatase was considered to be representative of all lysosomal hydrolases within the irradiated corneal epithelium. Non-irradiated or control corneas showed a uniform, diffuse stainability throughout the cornea. The target zone within the irradiated corneas, which were fixed immediately after treatment, often showed a small positively-stained spot measuring 1 mm in diameter, although approximately half of the irradiated corneas showed no observable increase in stainability. All of the irradiated corneas harvested 24 hours after treatment showed a dense brown stain within the target area which varied in diameter ranging from 1.5 mm to 4.5 mm. At 48 hours post-

irradiation, however, the staining reaction within the target area was weak and diffuse and sometimes barely visible. The diameter of these lesions ranged from 2 mm to 4 mm. These data suggested that lysosomal hydrolases, as represented by acid phosphatase, increased within the irradiated zones with a peak staining intensity at 24 hours. By 48 hours, the staining reaction suggested that the tissue was in the process of recovering to normal levels of lysosomal enzyme activity.

The data acquired during this study suggested that the CO₂ laser at threshold power densities could produce increased concentrations of lysosomal enzymes within the corneal epithelium. These hydrolases could perfuse into the surrounding cytoplasm of the epithelial cells and the cathepsins within this complex could inactivate lactic dehydrogenase as well as other enzymes.

IV. Wavelength-specific laser damage

A. The effect of the ruby laser on pigmented cells.

The earliest studies with laser energy showed that the pigmented areas of skin on experimental animals was more responsive to ruby laser treatment than nonpigmented areas. We also observed that cells in tissue culture containing melanin granules showed injury and death upon exposure to laser energy. These included pigmented human skin, mouse and hamster melanoma, and retinal epithelium from human, rabbit, and chick sources. Low energy densities (15 j/cm²) produced cytoplasmic retraction and pyknosis of the nuclei. Higher values (1000 j/cm²) produced immediate cellular death. Nonpigmented cells, derived from human skin, mouse lung, a nonpigmented hamster melanoma, and an albino rabbit retinal epithelium, showed no morphological changes following exposures to laser energy up to 1000 j/cm². These elements were observed to be viable and to exhibit mitotic figures for at least 48 hours after radiation.

The outgrowth of retinal epithelium frequently showed a cell population with a variable degree of pigmentation. When such a mixed population was exposed to laser energy, the effect was proportionate to the degree of pigmentation. Human retinal epithelial cells showed a percentage of injury and death that was correlated with the number of pigment granules per cell present at the time of radiation.

These data indicated that biological damage was observed only when laser energy was absorbed by a chromophore, such as melanin granules. In theory, different laser wavelengths would have different effects on cellular components, because laser energy would be absorbed by natural chromophores in cellular organelles only if their absorption characteristics matched the imposed laser wavelengths. The remainder of this section summarized the types of cellular

or biochemical changes recorded after treating tissues with laser energy ranging from visible to far ultraviolet wavelengths, as well as a near infrared wavelength.

B. Hemoglobin as a specific chromophore for green wavelengths.

A study was made of the response of hemoglobin in solution and in intact erythrocytes following exposure to either Q-switched ruby laser power, at 6943A, or a frequency doubled neodymium laser wavelength at 5300A. The two laser systems had comparable pulse widths and power densities but varied in wavelength. Hemoglobin was selected because it absorbs optimally in the green region of the spectrum, but transmits red light.

Repeated exposure of oxygenated hemoglobin solutions to 1 MW of green laser power from one to five times produced no detectable effect. However, a similar procedure altered the shape of the absorption spectrum of the reduced hemoglobin to one which was intermediate between the reduced and oxygenated curves.

Unirradiated red blood cells on an unstained, air-dried slide showed the typical biconcave morphology of the human erythrocyte. Within the target area of the focused green laser beam (110 MW/cm²), the erythrocytes were seen to have intact membranes and appeared somewhat flattened although the concave appearance was retained by many cells. Several others showed the typical morphology of the "target cell" found in many hypochromic anemias, but particularly in Cooley's anemia. Although the variable surface configuration of the red blood cell led to non-reproducible measurements with an interference microscope, no appreciable difference in mass was observed.

The cytophotometric measurement of individual cells in the region of 5000A to 6000A indicated an insufficient optical density (OD) to describe their state of oxidation or reduction. However, the presence of hemoglobin was demonstrated in isolated control cells by maximum OD readings of 0.3 to 0.4 observed at 4150A. Cells close to the edge of the target area showed lower optical density values, while irradiated cells throughout the bulk of the target showed no capacity to block light in a 4000A to 6000A range. The application of light from a Q-switched ruby laser at an equivalent power density showed no such change in light absorption by the cells.

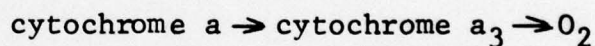
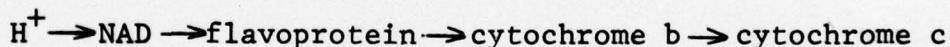
C. Cytochromes as chromophores for orange and green wavelengths.

Freshly prepared suspensions of newborn rat brains,

monitored with a physiological gas analyzer, revealed a high rate of oxygen consumption. It was observed, however, that the slope of the tracing could be altered by exposing the suspension to multiple flashes of 1 MW/cm² each of green (5300A) laser power derived from a frequency doubled neodymium laser system. The rate of oxygen consumption was thus suppressed by 30-40% of control values. A replicate experiment, using a Q-switched ruby laser of equivalent pulse width and power density, produced no detectable respiratory inhibition.

It was postulated that if green laser power could inhibit oxygen consumption but a wavelength of 6943A could not, the reduced cytochrome c, with absorption maxima at 5200 and 5500A, might have served as a natural chromophore. A control aliquot of newborn rat brain cell suspension was oxygenated and placed in a reference spectrophotometer cuvette. A second aliquot which had sustained 10 exposures to 1 MW/cm² each of green laser power was similarly oxygenated and placed in the "sample" cuvette. The difference spectrum, as recorded by the Beckman DK-2 spectrophotometer, revealed that all of the cytochromes in the radiated sample became oxidized except cytochrome b.

Specific cytochromes are known to act sequentially to transfer electrons and associated hydrogen ions (H⁺) to the terminal cytochrome where the H⁺ is bound with elemental oxygen to form water:



The fact that cytochrome b remained in a reduced state despite the presence of oxygen indicated that cytochromes c + c₁ failed to act as electron acceptors. The green laser frequency appeared to produce a block in this metabolic pathway.

The response of brain cell suspensions to treatment with wavelengths of 6096 and 6013A, derived from a Raman-shifted ruby laser beam, tended to confirm this conclusion. The difference spectrum resulting from this treatment indicated that cytochromes b, c, and c₁ remained reduced in the presence of oxygen, which revealed the failure of cytochrome a to act as an electron acceptor. Thus, a metabolic block was established between cytochromes c and a when the laser frequency was matched to the frequency range absorbed specifically by cytochromes a + a₃.

Finally, it was established that the cytochrome-rich sarcosomes in myocardial cells in tissue culture were sensitive to the 5145A wavelength of 1 watt pulsed argon ion laser when focused onto the organelle through the optics of a phase-contrast microscope. Performations and charred

lesions could easily be induced in sarcosomes, but other areas of the cell appeared to be resistant to argon laser treatment.

D. Artificial Chromophores for visible wavelengths.

The constituents of the chromosomes deoxyribonucleic acid (DNA) and nucleo-proteins, do not absorb visible light. It was therefore necessary to stain the chromosomes with a photosensitizing agent, acridine orange. In very dilute solutions this dye, which binds itself directly to the DNA, did not seem to be toxic to cells. Cells were exposed to a 10^{-5} or 10^{-6} percent solution of the dye for five minutes and were then washed several times with saline solution. These cells divided normally in tissue culture and were not significantly different in appearance from control cells.

When acridine orange-stained salamander lung cells were treated with the focused argon ion microbeam laser, we found it possible to produce lesions of various sizes by changing the concentration of dye, the output of the laser or the optics of the microscope. With a more concentrated solution of acridine orange (2.5×10^{-3} percent) distinct pieces of chromosomes could be broken off. A chromosome could also be cut in half by firing the laser several times across it. More dilute concentrations of dye produced a lesion that appeared as a pale spot on the chromosome.

The structural lesions (as indicated by a phase-pale zone which was negatively stained for DNA with the Feulgen reaction) were also shown to be functionally deficient. For example, if the nucleolar organizer region was damaged by laser treatment, it failed to form a nucleolus when the dividing cell entered telophase.

As in the chromosome studies, it was necessary to sensitize the nucleoli to the laser light. This was achieved by treatment with the antimalarial drug quinacrine hydrochloride. The steps in the irradiation process were slightly different from the chromosome sequence. Unlike acridine orange, quinacrine can easily be washed out of cells; therefore the drug must be present in the culture chamber during irradiation. If the quinacrine was washed out immediately after irradiation and fresh culture medium was placed in the chamber, the cells continued to grow. The morphology of the lesions was remarkably consistent with the morphology of the nucleolus: a lesion placed in a dense nucleolar region gave the appearance of a hole, whereas lesions made in less dense regions appeared as distinct dark spots.

Functionally, cells with these kinds of nucleolar lesions showed a reduced capacity to incorporate ^3H uridine, as seen by autoradiography.

E. Reduced nicotinamide adenine dinucleotide as a chromophore for near ultraviolet wavelengths.

The beam from a Q-switched ruby laser was directed through an ammonium dihydrogen phosphate crystal, which converted 20% of the chromaticity from 6943A to 3472A. This ultraviolet emission was separated from the red wavelengths by passing the mixture through a prism. The maximum ultraviolet energy achieved by this technique was 0.1 joule, which was distributed over about 0.78 cm^2 during a 30-nanosecond emission time. A variety of cell types derived from human lung, thyroid, and kidney, and from rabbit endothelium were exposed to the 3472A and 6943A wavelengths, singly or in combination. The imposed energy was modified either by using multiple flashes or by partially focusing the beam through a biconvex lens.

Unpigmented cells in tissue culture generally showed cytoplasmic blebbing within 10 min. after exposure to 0.125 j/cm^2 of 3472A laser energy. Within 1 hr. a number of cells in the radiated area showed pyknosis of the nucleus and nucleolar vacuolation. After 72 hrs, all of the cells in the target area had died and fallen off the glass. In contrast, those cells treated with a ruby laser wavelength of 6943A showed no apparent morphological change.

Nicotinamide adenine dinucleotide (NAD) is a coenzyme which participates in a number of vital biochemical reactions in living cells. In its oxidized form, NAD absorbs only in the far ultraviolet region of the spectrum, but its reduced form (NADH) shows an additional absorption peak at 3400A. Therefore, we postulated that this molecule might serve to selectively absorb the 3472A laser wavelength, but not the 6943A wavelength. Therefore, we tested the possibility that the near ultraviolet energy might inhibit the lactic dehydrogenase (LDH)-catalyzed conversion of pyruvate plus NADH to lactate plus NAD. Components of the reaction mixture were exposed singly to laser power at 3472A, then mixed with the other components. The rate at which NADH was oxidized was studied by recording the rate of change in optical density of the solution at 3400A with a Cary spectrophotometer.

There was no difference in the reaction rate between unirradiated mixtures, laser-treated NADH and laser-treated LDH solutions. However, when NADH and LDH were mixed and exposed to the 3472A laser power, we observed a 60% reduction in the reaction rate. These data suggest

that NADH absorbed the laser energy at 3472A and transferred the captured energy to the LDH molecule, resulting in a partial inactivation of the enzyme.

F. Lactic dehydrogenase as a chromophore for a far ultraviolet wavelength.

Although we observed that lactic dehydrogenase (LDH) activity was not affected by laser irradiation at 3472A its ultraviolet absorption characteristic suggested that a far ultraviolet laser wavelength may cause inhibition of enzymatic activity. To test this hypothesis, we used a neodymium doped, Q-switched laser (Korad, Model KIQP) with a primary output of 125 MW in the infrared region of the spectrum (10,600A), and half pulse width of 15 nsec. A potassium dihydrogen phosphate crystal was inserted in the cavity to act as a frequency doubler to generate the second harmonic at a wavelength of 5300A with an efficiency of 8 to 10 percent. This green light was transmitted through a mirror which was 100 percent reflective to the infrared and was passed through a second doubling crystal with a conversion efficiency of 1 to 2 percent. The converted ultraviolet wavelength at 2650A was transmitted through a quartz filter, which was 100 percent absorbent for the 5300A. Utilizing the malachite green leucocyanide actinometric technique, we found that a power density of 150 to 180 kW of the far ultraviolet wavelength was emitted in a 15-nsec pulse width. A helium-neon alignment laser was utilized to indicate the position of the ultraviolet beam in order to properly orient the target. The oxidation of reduced nicotinamide adenine dinucleotide (NADH), in the presence of pyruvate and LDH, was used as an assay system.

After establishing a control enzymatic reaction rate at room temperature, a portion of the LDH enzyme (0.05 ml) was placed in a quartz spectrophotometer cuvette and positioned in the path of the ultraviolet laser (UVL) beam. The laser beam was focused with a quartz lens through the front surface of the cuvette into the LDH solution. The enzyme samples received from 30 to 60 laser pulses. Immediately after the last exposure of the enzyme to the laser power, 2 ml of the substrate solution was added to the cuvette, and the rate of NADH oxidation was monitored spectrophotometrically. An additional unirradiated sample of LDH that was maintained at the same temperature as the irradiated sample was evaluated for catalytic activity after the enzyme samples were laser irradiated.

It was apparent that the ultraviolet laser power affected the enzymatic activity of LDH. Furthermore, it appeared that the degree of inactivation was proportional to the amount of ultraviolet radiation power imposed, since the portion that received 60 pulses took approximately twice as long to run to completion as the portion that

received 30 pulses. Since other enzymes show similar absorption characteristics due to their proteinaceous nature, it can be assumed that they would be inhibited by the UVL treatment as well.

G. DNA as a chromophore for a far ultraviolet wavelength

Chinese hamster cells (DON-C pseudodiploid strain) were exposed to multiple flashes of 2.3 mJ/cm^2 from a frequency-quadrupled neodymium laser operating at 2650A. This form of laser treatment produced all known types of chromosome and chromatid aberrations. They included chromatid and chromosome deletions, chromosomal translocations, dicentric, tricentric, ring chromosomes and minutes. The frequency of these aberrations was greatest in populations exposed to 2.3 mJ/cm^2 , due to the larger number of suitable cells which were available for analysis. Cells exposed to 11.5 mJ/cm^2 offered few mitotic figures, which generally showed clumped or fragmented chromatin masses.

A parallel study was conducted to determine the effect of the UVL on template activity of calf thymus DNA. The DNA solution was extruded from the siliconized tip of a micropipette in 0.015 ml drops which were irradiated while clinging to the pipette tip through surface-tension forces. Following multiple exposures of the drops to a focused UV laser beam at room temperature, the irradiated DNA was carefully collected and mixed with 0.3 ml of an incubation mixture, which consisted of ^{14}C -UTP (920 uCi/umole), 0.15 umole, ATP, CTP, GTP, each 0.15 umoles, MgCl_2 , 0.3 umoles, MnCl_2 , 0.03 umoles, 2-mercaptoethanol, 0.03 umoles, and *E. coli* RNA polymerase (bio-polymer product), 5 ug. After an incubation period of 30 minutes at 35°C , the reaction was terminated by adding trichloroacetic acid, and the resultant precipitate was collected on a membrane filter. The radioactivity of the incorporated ^{14}C -UMP was counted in a gas flow counter.

RNA synthesis, as indicated by ^{14}C -UMP uptake, was inversely proportional to the imposed laser energy. The unirradiated control DNA promoted the incorporation of 540 p moles of UMP into newly synthesized RNA, but this diminished exponentially to 129 p moles UMP when the DNA was treated with a graded series of energy densities to a total of 65 mJ/mm^2 laser energy at 2650A.

The rate of DNA and RNA synthesis and the percent of DON-CII cells entering DNA and RNA synthesis was also found to be depressed after ultraviolet laser irradiation. The number of DON-CII cells in DNA synthesis were recorded after pulse labelling with ^3H -TdR following 690 ergs/ mm^2 and 1150 ergs/ mm^2 . A decrease in the uptake of ^3H -TdR was observed after both doses over a 6-hour period, and the effect was greatest with the higher dose. The maximum effect was seen 4.5 hours after exposure and was 82 percent of the control after 690 ergs/ mm^2 , and 70 percent

after 1150 ergs/mm². The effect diminished with time and returned to control values 24 hours after 690 ergs/mm²; however, it remained depressed by 24 hours (84 percent of controls) after 1150 ergs/mm². The average number of grains per cell for the same doses were enumerated, and it was found that there was a significant decrease in the irradiated cells up through 3.5 hours after exposure compared to controls; however, there was a return to control values by 6.5 hours. The maximum decrease was seen 2.5 hours after exposure for both doses. There was very little difference between the numbers of the grains per cell between the two doses.

The rate of RNA synthesis in DON-CII cells was determined by the incorporation of ³H-UdR (pulse labelling) after 1150 ergs/mm². There was about a 50 percent reduction in the number of cells utilizing ³H-UdR, 3 and 4 hours after irradiation compared with the controls. However, by the 5th hour a 25% increase was observed, and a return to control values was reached by 7 hours. Grains per cell decreased to between 50 percent and 60 percent of the controls, 4 and 5 hours after irradiation followed by an increase thereafter, reaching a plateau (74 percent) by 7 hours and remaining at this level (75 percent) 24 hours after exposure.

H. Nonpigmented cells transmit energy in the near infrared region

The normal diploid human fibroblast, WI-38, was used to study the effect of multiple pulses from a Q-switched neodymium (Nd) laser with a power output of 15 MW/pulse. Several replicate studies showed that 0-40 pulses of laser power at 10,600A had no measurable effect on the growth rate of these cells over a three-day interval.

Autoradiographs were prepared for control cells and for cells incubated 2, 4, 6, 8 and 24 hours after receiving 40 pulses from the 15 MW Nd laser. Laser treatment at 10,600A had no significant effect on the percent of labelled cells or the number of grains over the cells, irrespective of whether the cells were incubated in ³H-thymidine, ³H-uridine or ³H-leucine. This demonstrated lack of effect on growth, DNA, RNA and protein synthesis supports the view that no chromophore exists for WI-38 cells which would absorb the 10,600A wavelength.

V. Two-photon absorption in biological systems.

As shown in the previous sections, the biological effects of laser energy are usually mediated through an absorption of the laser frequency by chromophores within tissues. However, some destruction of biological materials

has been reported, when either large energy sources, Q-switching techniques or critical focusing was utilized, which could not be explained on the basis of matching the input laser frequency with the absorption characteristic of the target.

The availability of extremely high photon densities from laser instrumentation has made it possible to demonstrate two-photon absorption in a number of organic solutions and crystals. If it could be assumed that organic components within the cell would also demonstrate this phenomenon, a partial explanation could be developed for observed damage other than that which is dependent upon a single photon absorption event.

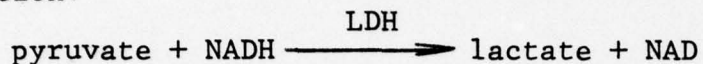
The reduced form of a nicotinamide-adenine dinucleotide (NADH) was selected as a model for study. It functions as a coenzyme in a number of important biochemical events within a cell. It can be activated with wavelengths around 3400Å to fluoresce, yielding measurable evidence of its excited state. A laser wavelength of 3472Å has been shown to limit the rate of a biochemical reaction in which it participates. Finally, its absorption characteristics suggested that two photons (28804 cm^{-1}) from a ruby laser might be absorbed, while a single photon (14402 cm^{-1}) absorption would be highly unlikely.

A preliminary study indicated that it was possible to excite fluorescence in an NADH solution, not only with the 3472Å wavelength, as expected, but also with the primary wavelength at 6943Å. It is generally considered that fluorescence intensity is dependent on the square of the incident intensity if a two-photon process is operational. To demonstrate this relationship, a series of photon intensity measurements were made. The red photons from a Q-switched ruby laser were passed through a Corning 2-58 filter and partially focused on an NADH solution (0.67 mg/ml). The fluorescent photons from the sample were passed to a S-20 photodiode through a Corning 4-104 filter, which transmitted in the spectral region of 4700-4800Å. It was noted that no blue photons could be detected from either water or a solution of the nonfluorescing NAD. However, by operating over a power density range from 3 to 18 MW, it was possible to measure increasing fluorescence intensities from laser-excited NADH.

A plot was constructed of the log of the fluorescent intensity versus that of the exciting intensity. A slope of 2 was found, corresponding to a square-law relationship between the intensity of fluorescence and incident intensity which indicated a double-quantum effect

While it was possible to demonstrate two-photon absorption in the reduced coenzyme, the question remained as to whether this event would merely produce a transitory excited state, or whether some molecular disruption would result which would lead to a decreased metabolic reaction rate and eventual cellular death.

The treatment of an NADH solution with only two pulses for laser light (58-60 joules) produced a partial inhibition in its participation as a coenzyme in the reaction:



Treatment of the enzyme, lactic dehydrogenase (LDH) with laser energy did not change the rate of the reaction, but laser exposure of an NADH solution alone or in the presence of the enzyme caused a significant decrease in the rate of the reaction.

A study was also conducted to determine if the two-photon absorption phenomenon could be detected in living tissues. Pigmented and albino rabbits were sacrificed, the eyes enucleated, and their irises carefully dissected to provide an intact ring of the iridial sphincter muscle with some adherent iris tissue. These muscle rings were looped over a pair of stainless steel wire hooks and suspended in Gey's balanced salt solution within a spectrophotometer cuvette. The lower hook was stationary, while the upper hook was attached to the suspended arm of a chemical balance. The opposite end of the balance arm was extended with an aluminum foil tube to which was attached a black paper mask. The mask moved vertically between a light source and a cadmium-sulfide photoconductor. The resulting variable electric signal was amplified and recorded with a strip chart recorder. A full-scale deflection of the pen could be made to represent a muscular excursion of 0.1 mm or less by adjusting the span setting of the recorder.

The laser energy source consisted of the MS-2 biomedical ruby laser, having an emission capability of 100 J and a pulse width of 2.5 msec. Utilizing a built-in aiming light, we could direct a partially focused laser beam (2 to 4 mm target diameter) onto different surfaces along the length of the stretched muscle loop. Each burst of laser energy was monitored with an internal calorimeter. The calculated energy density imposed on the surface of the tissue ranged from 100 to 1000 J/cm². Upon impact with a laser beam, there was a localized contraction within the target area. Relaxation of the muscle to its original position occurred within 1 to 4 minutes, depending on the amplitude of the contraction.

A comparison of the relative responses of excised pigmented and albino rabbit irises indicated that the pigmented tissue contract at lower laser threshold values and with greater excursions than did the tissue from albino rabbits. On the assumption that the pigmented tissue contracted in direct proportion to the laser energy absorbed, by virtue of the absorption characteristics of the pigment granules, its dose-response relationship was plotted to show a slope of 1.0. Using the same coordinates, the dose-response of the albino tissue showed a slope of 2.0. As with the NADH fluorescence intensity study, this slope was considered to be indicative of a double-quantum effect.

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